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ISOLATION AND CHARACTERIZATION OF MICROBIAL IMMUNOGLOBULIN A PR--ETC(U)
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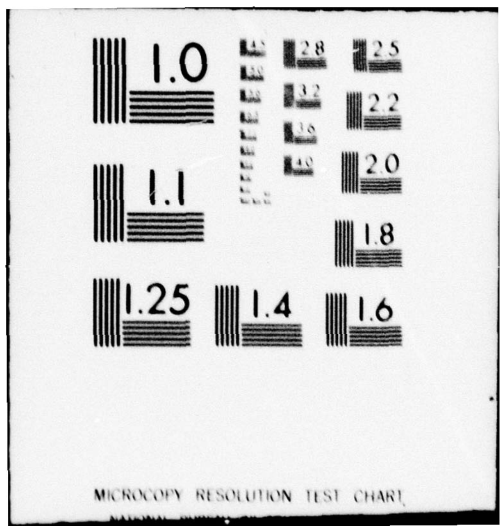
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(6) ISOLATION AND CHARACTERIZATION OF MICROBIAL IMMUNOGLOBULIN A PROTEASE.

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Progress Report

Contract No. DAMD-17-74-C-4022

Period covered by report: Jan 1 - Dec 31, 1976

Principal Investigator: Andrew G. Plaut, M.D.

During the calendar year encompassing this report, we feel that substantial progress has been made in characterizing the IgA protease of the pathogenic neisseria, and in demonstrating its effects on human antibody populations of the IgA class.

Having earlier verified that human IgA1 subclass molecules are uniquely susceptible to cleavage by gonococcal IgA protease, we undertook to establish methods for quantitation of the IgA subclasses in human secretions.

The technical difficulties one encounters in such work can be briefly listed as follows:

1) Variability of pH, salt composition and other biochemical constituents of secreted fluids under study

2) Heterogeneity of secretory IgA (the substance to be quantitated) with request to its amino acid sequence, tendency to polymerize into chains of varying length, and presence of secretory component on the molecule

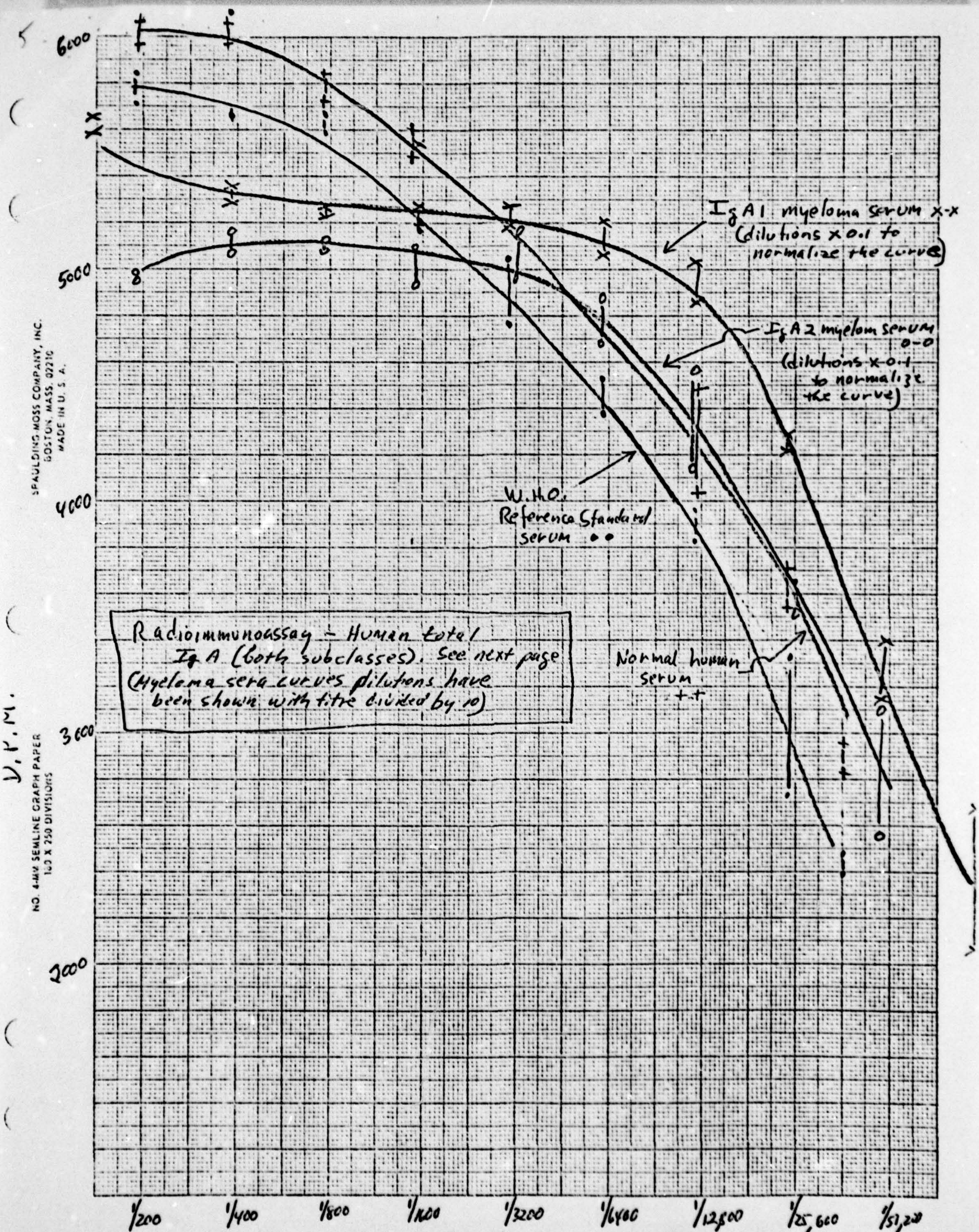
3) Minute quantities of IgA in the secretions under study, usually present in microgram amounts per ml.

We elected to begin this work by establishing radioimmuno assay (RIA) for the two subclasses of human IgA.

A considerable amount of preliminary work has already been accomplished in our laboratory in establishing this assay. The RIA is based on antigenic differences between the IGA1 and IgA2 subclasses which arise because of the deletion in the hinge region of the IgA2 heavy chain. Studies using Ouchterlony double diffusion analysis developed with unabsorbed rabbit or goat anti-IgA1 antiserum reveal that IgA1 paraproteins invariably "spur over" IgA2 paraproteins, a reflection of the antigenic dominance of IgA1. With relatively little difficulty such an anti-IgA serum can be rendered specific for the IgA1 subclass by absorption with purified IgA2 proteins. The resulting serum detects only IgA1 immunoglobulins and probably does so because the antiserum is now specific for the hinge region difference between IgA1 and IgA2. The converse situation, in which an anti-IgA serum is absorbed by IgA1 proteins thereby rendering it IgA2-specific has not yet been achieved in our laboratory, and to my knowledge has only been rarely accomplished by others. The difficulty lies in the paucity of antigens unique to IgA2 proteins when compared to IgA1. We attempted to prepare IgA2 specific antisera by immunization of a variety of animals by purified IgA2 in various dosage schedules. A high titer anti-human IgA1 serum has been prepared and we now have the potential for assay of the total IgA content and, specifically, the IgA1 in biological fluids. The difference in values will be attributed to IgA2 until a specific antiserum which can identify the second subclass is obtained. There is at present no evidence that more than two IgA subclases occur in human beings.

RIA involves a double antibody technique in which test samples compete with purified ^{125}I labelled IgA subclasses for precipitation by goat antisera. The second antibody is rabbit anti-goat gammaglobulin. For the assay of total IgA levels the first antibody is goat anti-human IgA raised to myeloma proteins; light chain specificity was removed by passage of this antiserum over IgA-free human serum bound covalently to Sepharose 4-B activated with cyanogen bromide. The absorbing serum, which has a near normal IgG content, was from a patient with ataxia teleangectasis who is totally lacking in serum IgA by conventional immunochemical criteria. The ^{125}I labelled protein in the assay is a human IgA2 myeloma. The reason for using this subclass in the assay for total IgA is because such a protein will have the antigenic sites common to both IgA1 and IgA2 subclasses and, conversely, will have none of the structural feature unique to IgA1. This insures that the first antibody has minimal, if any, tendency to bind to one (the IgA1) subclass preferentially. We have already demonstrated that IgA1 and IgA2 myelome paraproteins are equally detected in the assay by performing antigen dilution curves with purified preparation of the two subclasses; the binding curves are virtually identical. This is an important precaution, since secretory and serum IgA are composed of mixtures of both subclasses and therefore a measure of total IgA content requires that each subclass be equally active in the assay. External labelling of IgA proteins with ^{125}I by the method of Hunter and Greenwood.

In contrast to the assay for total IgA, the assay for IgA1 is determined in aliquots of the same biological samples by the double antibody technique using the aforementioned goat anti-human IgA1 antiserum. The labelled protein in this IgA1 assay is a purified IgA1 myeloma protein. To insure that the anti-serum is in fact specific for IgA1 it is absorbed by passage over purified human IgA2 myeloma proteins bound in solid phase covalently to Sepharose 4B. In preliminary experiments this antiserum is demonstrably IgA1-specific, since a panel of four purified IgA2 paraproteins at concentration of 2 mgm/ml were not detected, and an intact human serum containing 20 mgm/ml IgA2 myeloma protein reacts minimally in the assay (see curve below). The total IgA and IgA1 assays are capable of detecting immunoglobulin proteins at levels of 50 ng. per ml. of serum. Pertinent precipitation curves documenting this are shown on the following pages. Radioactive counting was by a Beckmann Biogamma II gamma counter. The legend for a given figure is found on the sheet which follows it.



6000
5000
4000
3600
2000
D.P.M.
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12,800
1/25,600
1/51,200
Serum dilutions
IgA1 myeloma serum x-x
(dilutions x 0.1 to normalize the curve)
IgA2 myelom serum o-o
(dilutions x 0.1 to normalize the curve)
W.H.O. Reference Standard serum • •
Normal human serum + +
Radioimmunoassay - Human total IgA (both subclasses). See next page
(Myeloma sera curves dilutions have been shown with titre divided by 10)
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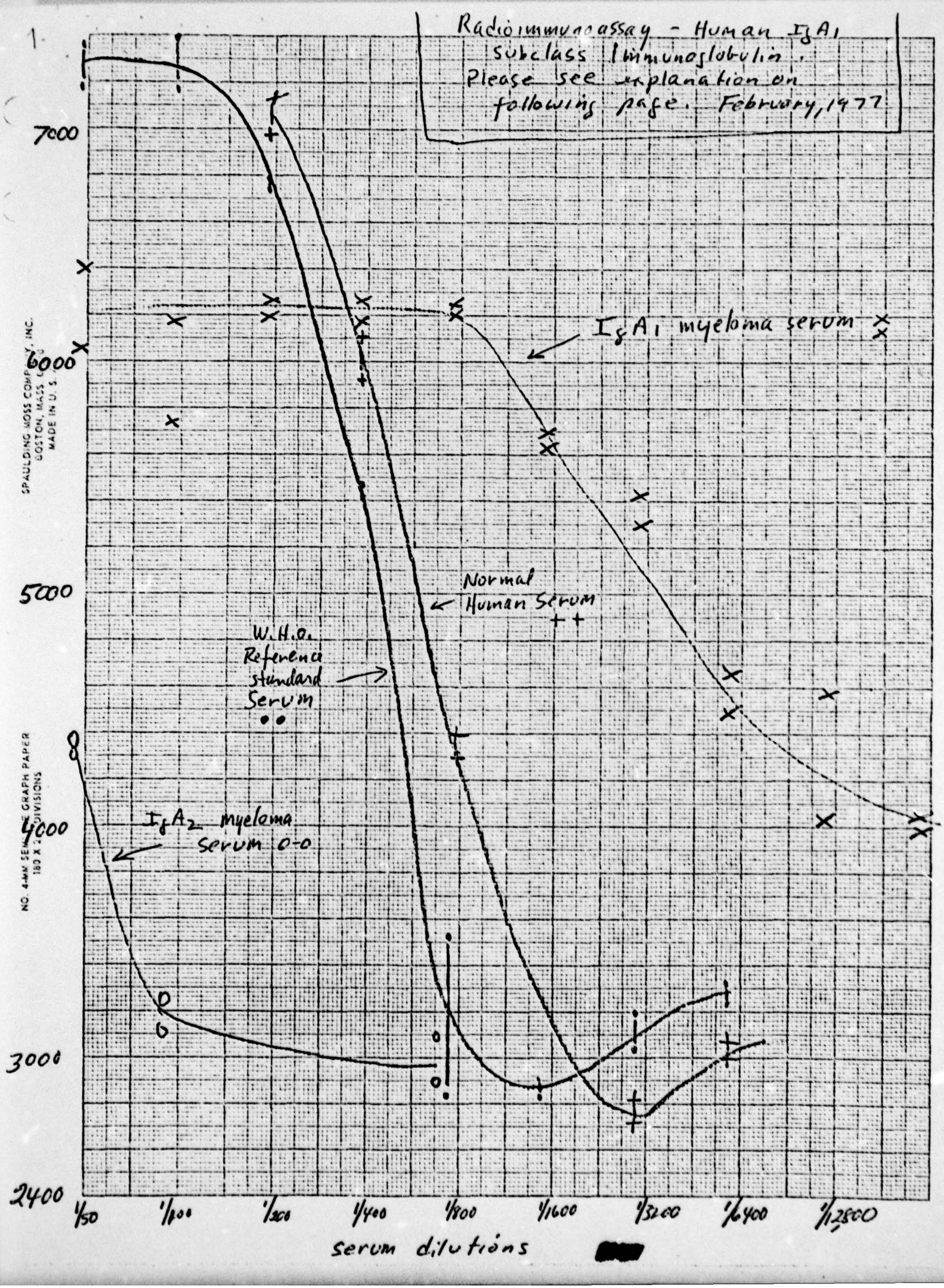
Legend to Preceding Figure

RIA, Hand-drawn curves with duplicate points shown, of total IgA assay in four sera: 1. Normal human serum. 2. W.H.O. Reference Standard for immunoglobulin quantitation. 3. Patient serum with IgA1 subclass myeloma paraprotein. 4. Myeloma serum, IgA2 paraprotein. Abscissa: serum dilutions (the myeloma sera are plotted at dilutions 10-fold less than actual value); ordinate: labelled IgA2 antigen remaining in solution after double antibody precipitation is complete.

The curves show that normal and myeloma sera, irregardless of subclass, are reactive in the assay. The lower values in myeloma sera at the 1/200 serum dilution point is discussed in the legend to the other figure.

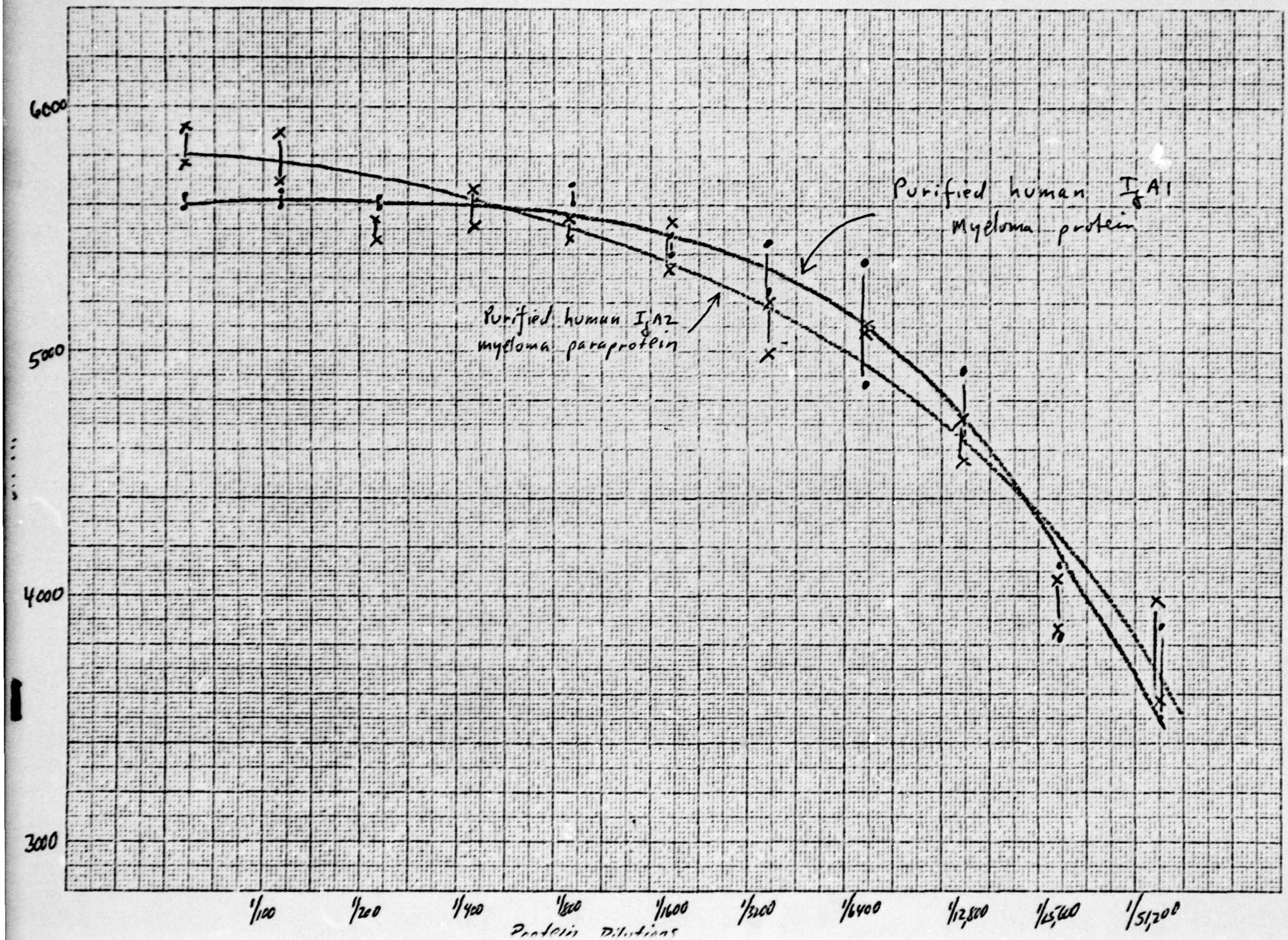


Radioimmunoassay - Human IgA1
subclass Immunoglobulin.
Please see explanation on
following page. February, 1977
7000
6000
5000
4000
3000
2400
IgA1 myeloma serum
Normal
Human Serum
W.H.O.
Reference
standard
Serum
IgA2 myeloma
serum o-o
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12,800
serum dilutions

Legend to Preceding Figure

Actual RIA, hand-drawn curves with duplicate points shown, of IgA1 assay in four sera: 1) Normal human serum, 2) W.H.O. Reference Standard for immunoglobulin quantitation, 3) patient serum with IgA1 subclass myeloma paraprotein, 4) Myeloma serum, IgA2 paraprotein. Abscissa: serum dilutions. Ordinate: labelled IgA1 antigen remaining in solution after double antibody precipitation is complete.

Curves show that standard and normal serum are comparable. IgA2 is nearly unreactive. IgA1 is detected. The explanation for lower starting counts (at low serum dilution) in IgA1 myeloma serum is unknown; this may represent polymerization of labelled IgA antigen with myeloma protein. We are working on this problem, which seems to be the only difficulty with the assay. It may be corrected by prior disulfide reduction of all reactants which will expose antigenic determinants and disassemble polymers.


6000
5000
4000
3000
Purified human IgA1
Myeloma protein
Purified human IgA2
myeloma paraprotein
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12,800
1/25,600
1/51,200
Protein Dilutions

Legend to Preceding Figure

RIA, hand-drawn curves with duplicate points shown, of two human IgA myeloma paraproteins purified and adjusted to the same protein concentration. Concentration was based on absorbency at 280 nM ($E^{1\%}_{cm}$ = 13.5). Assay was for total IgA content.

Note that the proteins do not differ significantly in their competition for the antibody, indicating that the total IgA assay will detect the two subclasses equally.

The standard serum on which quantitation is based is the World Health Organization Standard (NCI Immunodiagnostics Reference Center, Springfield, VA) Which contains 95.3 International Units IgA per ml. A pool of normal sera in our laboratory have been assayed against the W.H.O. Standard so that we can have available sufficient material to use as a control in each assay.

The W.H.O. Standard was also used to establish subclass standards as follows: we assumed that sera containing IgA1 or IgA2 subclass proteins from patients with multiple myeloma contains that subclass only. In essence, a serum with IgA2 paraprotein is considered to have no IgA1 and to be 100% IgA2 in type. Given the massively elevated levels of IgA in myeloma, and the suppression of other classes and subclass of immunoglobulin characteristic of myeloma, we believe this assumption to be reasonable and in error only by several percent. Since the assay for total IgA detects both subclasses equally, and the IgA1 assay does not significantly detect IgA2, the myeloma sera represent "pure" preparations of the two subclasses in serum for comparison to the W.H.O. Standard. This method will eventually be verified by the use of purified paraprotein of both subclasses whose concentration is determined by absorbence at 280 nM ($E^{1\%}_{280} = 13.5$).

Since gonococcal IgA protease cleaves IgA to yield an intact antigen binding Fab fragment the question arose as to whether antibody activity would, in fact, be lost when the enzyme attacked IgA. We have examined this point using 5 human IgA1 myeloma paraproteins having defined antibody activity, and all 5 lost virtually all antigen-binding ability following short-term exposure to gonococcal IgA protease. By contrast, human IgM paraproteins with antibody activity were not influenced by enzyme treatment. This is shown in the following table:

Serum	Para-Protein Class	Test System	Initial Reciprocal Titer	Reciprocal Titer after IgA Protease Treatment	Reciprocal Titer After Heated IgA Protease Treatment	Effect of Enzymes On Antibody Activity
Chr	IgA	latex-IgG	5, 120	40	5,120	Marked Decrease
		SRBC-IgG	20, 480	320	20,480	"
Dun	IgA	latex-IgG	5, 120	20	5,120	"
Boy	IgA	SRBC-IgG	1, 280	80	1,280	"
Fin	IgA	SRBC	163, 840	0	163,840	"
Rob	IgA	Human RBC	5,000, 000	20,480	5,000,000	"
Kas	IgM	latex-IgG	3,000	3,000	3,000	No Effect
Lay	IgM	SRBC-IgG 4^{0}C	4,096	4,096	4,096	"
Lat	IgM	latex-IgG	6,000	6,000	6,000	"
Fag	IgM	latex-IgG	6,000	6,000	6,000	"
Rheumatoid Arthritis		latex-IgG	900	800	800	"
		SRBC-IgG	5, 120	2,560	5,120	Minor Reduction

In an extension of this work we were unable to demonstrate binding of the Fab fragments, as shown indirectly by the inability of such fragments to block agglutination by subsequently added intact antibodies, and directly by negligible binding of isotopically labelled Fab. The loss of antibody activity of IgA Fab even though the Fab fragment is released intact probably relates to the requirement for the polymeric state for adequate functional antigen affinity of the IgA molecule. It is, therefore, important to emphasize that a free Fab fragment, even with binding site structurally intact, is likely to be several orders of magnitude weaker in functional affinity than its parent antibody.

To summarize, the years' work involved two main approaches to understanding the biological role of IgA proteases. 1) Its effect on antibody activity in human antibodies of IgA isotype and 2) the distribution of IgA subclasses in human secretory fluids.

The principal investigator hereby wishes to thank the USA R & D Command for their willingness to support our research.

Publications arising from this work are as follows:

1. Plaut, A.G., Gilbert, J.V., Artenstein, M.S., and Capra, J.D.: Science 190: 1103-1105, 1975

2. Doellgast, G.J. and Plaut, A.G.: Immunochemistry 13:135, 1976

3. Plaut, A.G., Gilbert, J.V. and Wistar, R. Jr. Infect. Immunity (in press)

4. Underdown, B.J., DeRose, J. and Plaut, A.G.: J. Immunol. (in press)

ABSTRACTS

1. Wistar, R., Plaut, A.G., and Gilbert, J.V. Clin. Res.24, 454a, 1976

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BOOK CHAPTERS

1. Plaut, A.G. Local Immunity in Gonococcal Infections. In Gonococcus, edit. by R. Roberts. John Wiley & Sons, Philadelphia. (in press)

Respectfully submitted,

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Professor Medicine

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